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Inhibition by singlet molecular oxygen of the vascular reactivity in rabbit mesenteric artery

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1 The effects of reactive oxygen intermediates derived from photoactivated rose bengal on the vascular reactivity have been evaluated in rabbit mesenteric artery ring preparations. The artery rings were exposed to xanthene dye rose bengal (50 nM) illuminated (6,000 lux) at 560 nm for 30 min. Spin trapping studies with 2,2,6,6-tetramethylpiperidine (TEMP) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) with electron spin resonance spectrometry were also conducted in solution (and not within tissues) to determine quantitatively the reactive oxygen species generated from photoactivated rose bengal.

2 Contraction of the ring preparations induced by noradrenaline (10^{-8} to 10^{-4} M) was attenuated by previous exposure to photolysed rose bengal; the observation that the pD_2 decreased without a significant reduction in maximum tension generation is consistent with the view that receptor dysfunction may be involved in the effect of photolysed rose bengal.

3 Prior exposure to photolysed rose bengal of the ring preparations inhibited the endothelium-dependent relaxation evoked by acetylcholine (10^{-6} M) and calcium ionophore A23187 (10^{-7} M), but not the endothelium-independent relaxation evoked by nitroglycerin (10^{-6} M).

4 A variety of scavengers, superoxide dismutase (33 units ml^{-1}), catalase (32 units ml^{-1}) and 1,3-dimethyl-2-thiourea (DMTU, 10 mM), which should eliminate the superoxide anion radical, H_2O_2 and the hydroxyl radical, had no effect on the attenuated responses to noradrenaline and acetylcholine induced by photolysed rose bengal. In contrast, the inhibition of the observed effect of photolysed rose bengal was obtained with addition of histidine (25 mM), a singlet molecular oxygen quencher.

5 It was found that photolysis of rose bengal from a 1:2:2:1 quartet, characteristic of the hydroxyl radical-DMPO spin adduct, which was effectively blunted by DMTU, superoxide dismutase and catalase whereas histidine was ineffective. The results of the electron spin resonance study also showed that a singlet molecular oxygen was produced by photoactivation of rose bengal; this was detected as singlet oxygen-TEMP product (TEMPO; 2,2,6,6-tetramethylpiperidine-*N*-oxyl). The formation of the TEMPO signal was strongly inhibited by histidine, but not by DMTU, superoxide dismutase and catalase.

6 It is suggested that the superoxide anion radical, H_2O_2 and hydroxyl radical are formed in addition to singlet molecular oxygen, and the data obtained from the present study indicate that singlet molecular oxygen is one of the most destructive oxygen species. Endothelium-dependent relaxation is quite vulnerable to singlet molecular oxygen. Singlet oxygen also depresses noradrenaline-induced contraction possibly via α-adrenoceptor dysfunction. This, in turn, may lead to vascular incompetence.

Keywords: Electron spin resonance spectrometry; endothelial cells; free radicals; singlet oxygen; vascular smooth muscle

Introduction

Oxygen-derived free radicals generated during reperfusion have been implicated in the pathogenesis of post-ischaemic tissue injury, with the vascular system often being the first target (Kontos *et al*., 1983; Hess & Manson, 1984; McCord, 1985). Several investigators have suggested that free radicals interfere with normal contractile function in vascular smooth muscle. Kontos and co-workers (1984) showed that free radicals produced an irreversible vasodilatation in pial arterioles of cats, *in vivo*. Similar findings were obtained by Okabe *et al*. (1983) in the cat mesenteric microcirculation. Xanthine oxidase-derived radicals were shown by Wolin and Belloni (1985) to decrease tension in noradrenaline (NA) precontracted vessel rings. Although these studies have examined the effects of oxidants on the vasculature, the species of reactive oxygen intermediates responsible for the vascular smooth muscle dysfunction remains unidentified.

A number of studies have shown that oxygen-derived free radicals, particularly the superoxide anion ($O_2^{\cdot -}$) and hydroxyl radical ($HO\cdot$) are generated after reperfusion of the ischaemic myocardium and are linked to the loss of contractile function (Zweier *et al*., 1987). Superoxide anion is relatively unreactive but is considered dangerous because its dismutation results in the formation of hydrogen peroxide (H_2O_2), which can potentially generate the highly reactive $HO\cdot$ radical in the presence of transition metal ions. It is therefore believed that ultimate tissue damage occurs because of the $HO\cdot$ radical.

Most studies have focused on $O_2^{\cdot -}$, H_2O_2 and $HO\cdot$ as biologically relevant reactive oxygen species and the mediators of vascular smooth muscle dysfunction (Wolin & Belloni, 1985; Rubanyi & Vanhoute, 1986; Todoki *et al*., 1992). Another reactive oxygen species is singlet molecular oxygen ($^1O_2$), which is not a radical. It is an electronically excited state of oxygen which results from the promotion of an electron to higher energy orbitals. Singlet oxygen is a short-lived species with a lifetime of 10^{-6} s and its energy above the ground state level is 22.5 kcal (Cadenas, 1988). No direct demonstration of $^1O_2$ formation *in vivo* has been published to date primarily due to two reasons: firstly, there are no specific traps available to measure $^1O_2$ generation; secondly, the very short lifetime of $^1O_2$ would make such a proof difficult. On consideration that $O_2^{\cdot -}$ and H_2O_2 are the least capable of producing vascular changes (see Rubanyi, 1988), the only species blamed for the injury is $HO\cdot$. Hydroxyl radical can initiate lipid peroxidation thereby

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producing lipid-free radicals which may become important sources of $^1O_2$ *in vivo* (Kalyanaraman *et al*., 1987; Loesser *et al*., 1991). So far, the effect of the $^1O_2$ scavenger histidine (Hearse *et al*., 1989; Kukreja *et al*., 1993; Ishibashi *et al*., 1996) on HO·-induced changes in vascular reactivity has been investigated (Todoki *et al*., 1992); but there is no study describing the effect of $^1O_2$. Therefore, the direct effect of $^1O_2$ *in vitro* on the functional reactivity of blood vessels seems to be of particular interest. This is the first study to evaluate the effect of exogenously generated $^1O_2$ from photooxidation of the rabbit mesenteric artery ring preparations, with rose bengal as a sensitizer, on their functional reactivity.

Methods

Vessel preparation and isometric tension recording

In accordance with our institutional Animal Care Committee guidelines, mesenteric arteries were taken from male albino (New Zealand) rabbits (1.5–2.5 kg) after exsanguination during anaesthesia with sodium pentobarbitone (50 mg kg^{-1}). Fat and other nonvascular tissues were gently removed from the blood vessels, which were cut into rings (2 mm width; the mean ± s.e.mean weight of the tissue was 5.64 ± 0.16 mg, $n = 237$), without disturbing the intimal layer, after immersion in ice-cold modified Krebs-Henseleit solution (mM: NaCl 118.0, KCl 4.7, $MgSO_4$ 1.18, $CaCl_2$ 2.5, KH_2PO_4 1.18, $NaHCO_3$ 25.0 and glucose 5.5, aerated with 95% O_2–5% CO_2; pH 7.2–7.3). The rings were suspended in a 10 ml water-jacketed tissue bath (37°C) with one end tied to a fixed point and the other to a force transducer (Nihon Kohden, JB-612-T, Tokyo, Japan); changes in isometric force were amplified (Nihon Kohden, AP-601-G, Toyko, Japan) and recorded (Nihon Kohden, PJ-691-G, Tokyo, Japan). Before the start of the experiment, the rings were allowed to equilibrate for 90 min in the modified Krebs-Henseleit solution which was changed at 15 min intervals. During this time, the rings were stretched to a passive tension of 1.5 g.

To inhibit completely the oxidation of noradrenaline (NA), ethylenediaminetetraacetic acid (EDTA) and ascorbic acid were added simultaneously to the bathing media (Furchgott, 1956); propranolol was also added to block β-adrenoceptors in most experiments.

Generation of $^1O_2$

Singlet oxygen was generated by photoexcitation of the light-sensitive dye rose bengal (50 nM), which is one of the most efficient sources of $^1O_2$ generation (Gandin *et al*., 1983). Rose bengal is a fluorescein derivative that absorbs light strongly in the range of 530 to 590 nm, with two major absorptions (due to the xanthene chromophore) at approximately 530 and 558 nm (depending on solvent and pH). It is tetraiodinate of 4,5,6,7-tetrachlorofluorescein converted to potassium salt. It has been used as a source for $^1O_2$ generation when irradiated by 560 nm light (6,000 lux, for 30 min). The sensitivity of these reactions together with the short half-life of $^1O_2$ and $O_2\cdot^-$ make rose bengal an ideal tool for the production of transient bursts of reactive oxygen intermediates. The system consisted of four cables; each delivering (530–590 nm) from a light source equipped with appropriate interference filters. The intensity of light delivered in the tissue bath was controlled by a rheostat (Kenko Co., Techno Light, KTS-150, Tokyo, Japan). The light intensity was measured by a lux meter (Topcon, IM-2D, Toyko, Japan).

Electron spin resonance (e.s.r.) analysis

In the reaction mixture (without the ring preparation) used for vessel reactivity experiment, the spin trapping studies were performed by mixing rose bengal (50 nM) with 100 mM or 40 mM spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) or 2,2,6,6-tetramethylpiperidine (TEMP), respectively, and illuminating for 30 min with 560 nm light (6,000 lux). Care was taken to keep the DMPO solution covered to prevent light-induced degradation. An e.s.r. detection of the spin adduct was performed at room temperature by a JES-RE3X, X-band, spectrometer (Jeol, Tokyo, Japan) at the following instrument settings: modulation amplitude, 0.25 mT; recording range, 5 mT; recording time 2 min; time constant, 0.03 s; microwave power, 8 mW (for DMPO) and 4 mW (for TEMP); and magnetic field, 335.6 ± 5 mT. To quantitate the spin adducts detected, the Mn^{2+} standard e.s.r. spectrum (MnO) was obtained.

Drugs

The following drugs and chemicals were used: acetylcholine chloride (ACh), calcium ionophore A23187, catalase, 1,3-dimethyl-2-thiourea (DMTU), histidine, (−)-noradrenaline hydrochloride, rose bengal and superoxide dismutase (SOD; from bovine blood, 3,300 u mg^{-1} of protein) (Sigma); ascorbic acid, EDTA and propranolol hydrochloride (Wako Chemicals, Osaka, Japan); TEMP (Aldrich); DMPO (Labotec, Tokyo, Japan; 99–100% pure, GC assay by Dojindo Laboratories, Kumamoto, Japan); and nitroglycerin (Nihon Kayaku, Tokyo, Japan). All agents except for A23187 were dissolved in distilled water. Calcium ionophore A23187 was dissolved in dimethylsulphoxide (DMSO) and diluted in modified Krebs-Henseleit solution before being added to the tissue bath (final concentration of DMSO: 0.7 μM; DMSO at this concentration has no ability to scavenge the HO· radical (Lee & Okabe, 1995)). All other reagents were of analytical grade.

Statistical analysis

Means and s.e.mean are given throughout. Two sets of statistical comparisons were made. Student's t test for paired samples was used when two populations were compared. Comparisons of subsequent intervention to controls were made by one-way analysis of variance, followed by Duncan's multiple-range test (Walpole, 1982). Differences were considered significant when $P < 0.05$.

Results

Figure 1 shows the experimental protocol for assessing the effects of incubation with 50 nM rose bengal alone or illumination with 6,000 lux green light for 30 min in the presence of rose bengal on NA-induced responses of the ring preparations. In all ring preparations examined, 1st test values (Figure 1, lane (i)) were not significantly different from each other, and the NA-induced response was stable (time-matched control, Figure 1a) during the experimental period. Furthermore, the illumination alone had no effect on this system (Figure 1b). Therefore, to simplify the presentation of data, 1st test values were used as controls, and the data of time-matched and illumination control values were not presented here. Figure 2 shows the effect of rose bengal with illumination for 30 min on the concentration-response curves to NA; the experimental conditions were as those depicted in Figure 1. As shown in Figure 2a, NA contracted the ring preparations in a concentration-dependent fashion; rose bengal alone was without effect. Photolysis of rose bengal depressed contractile responses to NA (Figure 2b). The pD_2 decreased from 6.22 ± 0.18 to 5.88 ± 0.16 ($P < 0.05$). Maximum tension development did not differ significantly between rings before and after oxidization by photolysed rose bengal (Figure 2b). Preliminary experiments demonstrated that illumination (6,000 lux) for 10 or 20 min in the presence of 50 nM rose bengal was ineffective on the concentration-response curves to NA (data not shown). NA at 2.58×10^{-6} M elicited approximately 50% of the maximum tension that develops in response to NA in the absence of rose bengal and illumination (control in Figure 2a).

We next determined whether or not the endothelium-dependent relaxation induced by ACh was altered by photolysed rose bengal. Relaxation responses to ACh were determined in rings contracted to a stable plateau tension by the addition of 2.58×10^{-6} M NA; the effect of photolysed rose bengal on the contractile responses evoked by NA was also assessed simultaneously. The experimental protocol of this series of experiments is shown in Figure 3. The responses to the 1st addition of NA and ACh (Figure 3, lane (i)) of the ring preparations tested were not significantly different from each other, and the responses were stable (time-matched control, Figure 3a; and illumination control, Figure 3b) during the experimental period in the present study. Therefore, 1st test values were used as controls, and the data of 3rd test values (Figure 3a, lane (iii)) were presented as recovery responses. The data are summarized in Figure 4. photolysed rose bengal significantly attenuated the responses elicited by NA (Figure 4a) and ACh (Figure 4b); the recovery responses were also depressed. When ACh was replaced by nitroglycerin, the relaxation response of the ring preparations to nitroglycerin was not affected by photolysed rose bengal (Figure 4c). The endothelium-dependent relaxation induced by A23187 (10^{-7} M) was attenuated irreversibly by photolysed rose bengal (Figure 5).

Photosensitization of rose bengal in the presence of oxygen leads to the generation of $^1O_2$ and $O_2\cdot^-$ (Paczkowski *et al.*, 1985; Lee & Rodgers, 1987). Superoxide generation, which can subsequently result in the production of H_2O_2 and HO·, can be regulated by SOD treatment, thereby decreasing the production of the HO· (Motohashi & Mori, 1983; Kukreja *et al.*, 1988). To determine whether it was $^1O_2$ or $O_2\cdot^-$-derived reactive oxygen intermediates that were responsible for the observed inhibitory effects, the role of oxygen radical scavengers in protecting the response to 2nd and 3rd tests to NA and ACh from photolysed rose bengal was examined. SOD, catalase and DMTU by itself had no effect on this system (Table 1). However, histidine (that has no effect on ACh relaxation by itself) potentiated the NA contraction (Table 1); phenylephrine (10^{-6} M)-induced tension was not affected (data not shown), suggesting that this scavenger may have a protective effect on the catecholamine from oxidation under the experimental conditions employed in the present study. Furthermore, histi-

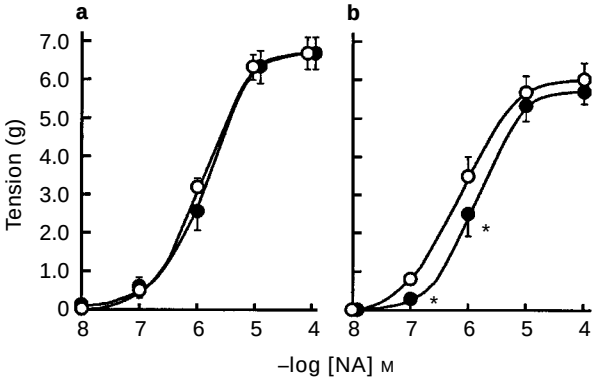

Figure 2 The effect of rose bengal with illumination for 30 min on the concentration-response curves to NA in rabbit mesenteric artery ring preparations. The experimental conditions were similar to those described in Figure 1. First test values in the rings treated with rose bengal alone (a) or rose bengal plus illumination (b) were used as controls (○), and compared with the values of 2nd test (●). Each point represents the mean, and vertical lines show s.e.mean ($n=6$); n refers to the number of rabbits from which the mesenteric artery was taken. $^*P<0.05$, significantly different from corresponding control values.

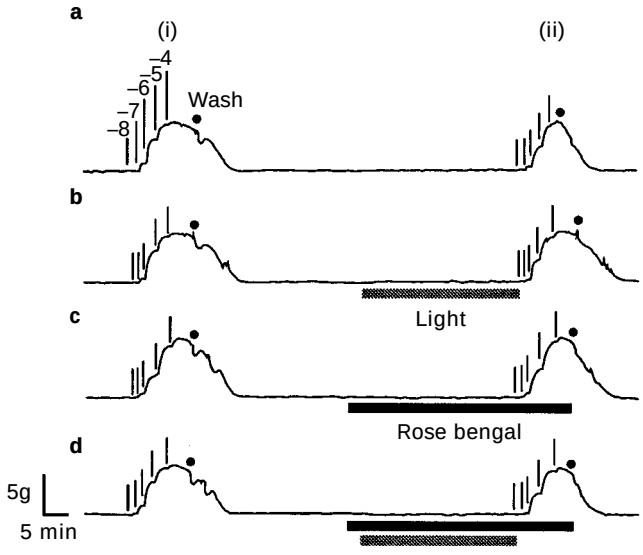

Figure 1 The effect of rose bengal and/or illumination for 30 min on the contractile responses to NA (10^{-8} to 10^{-4} M) of rabbit mesenteric artery ring preparations in the presence of 3×10^{-6} M propranolol, 2.6×10^{-5} M EDTA and 0.5 mM ascorbic acid (added 10 min before the addition of NA). Concentration-response curves were determined by the method of stepwise cumulative addition of NA in the absence (i) or presence (ii) of rose bengal. Four ring preparations (for time-matched control (a); illumination control (b); rose bengal alone (c); and rose bengal plus illumination (d)) obtained from the same vessel were studied in parallel. Rose bengal (50 nM) was added 2 min before the illumination (6,000 lux). Tracings are representative of six experiments. Numbers indicate the logarithm of the concentration (M).

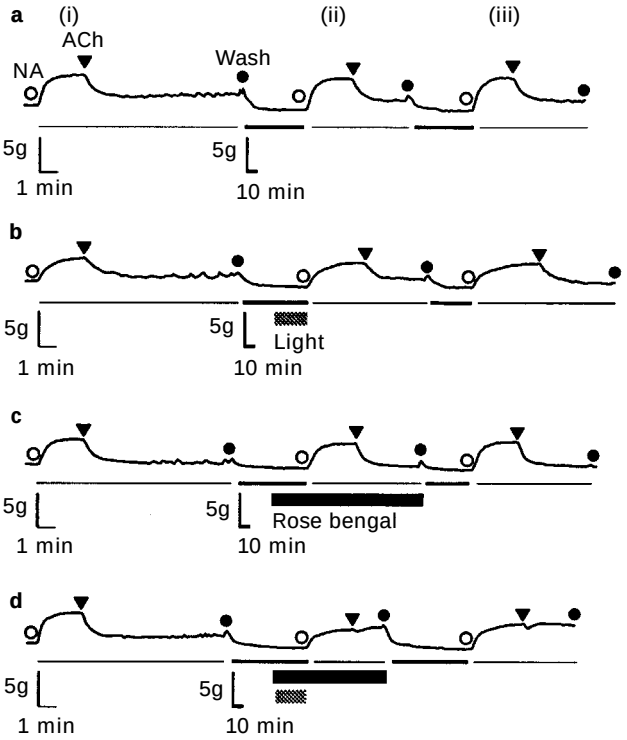

Figure 3 The effect of rose bengal and/or illumination for 30 min on the NA-induced contraction and ACh-induced relaxation responses of rabbit mesenteric artery ring preparations. The experimental conditions were essentially similar to those described in Figure 1 except that NA-induced active tone (NA, 2.58×10^{-6} M) and relaxation responses to 10^{-6} M ACh (at a stable plateau tension induced by the addition of NA) were determined in the absence (i) or presence (ii) of rose bengal; recovery responses to NA and ACh (iii) were also determined. Four ring preparations (for time-matched control (a); illumination control (b); rose bengal alone (c) and rose bengal plus illumination (d)) obtained from the same vessel were studied in parallel. Tracings are representative of five experiments.

dine, but neither SOD, catalase nor DMTU, protected against the responses to 2nd and 3rd tests to NA and ACh (Table 1); the observed effect of photolysed rose bengal on the A23187-induced relaxation was also protected against by histidine (Figure 5).

In a further attempt to determine whether $^1O_2$ is involved in the vascular dysfunction, the generation of $^1O_2$, $O_2\cdot^-$ and HO· was verified by e.s.r. spectroscopy with TEMP and DMPO as the spin trap in our system. Photooxidation of TEMP has been used for detecting $^1O_2$ production (Lion *et al.*, 1976). The product of this reaction has been shown to be TEMPO (a stable nitroxide free radical) that gives a typical three-line e.s.r. spectra in X-band. The characteristic e.s.r. spectral pattern of three equal-intensity lines was observed when rose bengal was illuminated for 30 min in the presence of TEMP (Figure 6b) under the same reaction conditions as those of the vessel reactivity experiments (but the ring preparation was omitted). The hyperfine splitting constant and g value of the radical was found to be $A_N = 0.172$ mT any $g = 2.0056$, consistent with values found previously (Lion *et al.*, 1976). Histidine (Figure 6c), but not SOD (Figure 6d) and catalase (Figure 6e), inhibited the TEMPO formation; DMTU (Figure 6f) was slightly effective. Incubation of TEMP with rose bengal without illumination (Figure 6a) or photolysis of rose bengal without TEMP did not produce TEMPO (data not shown).

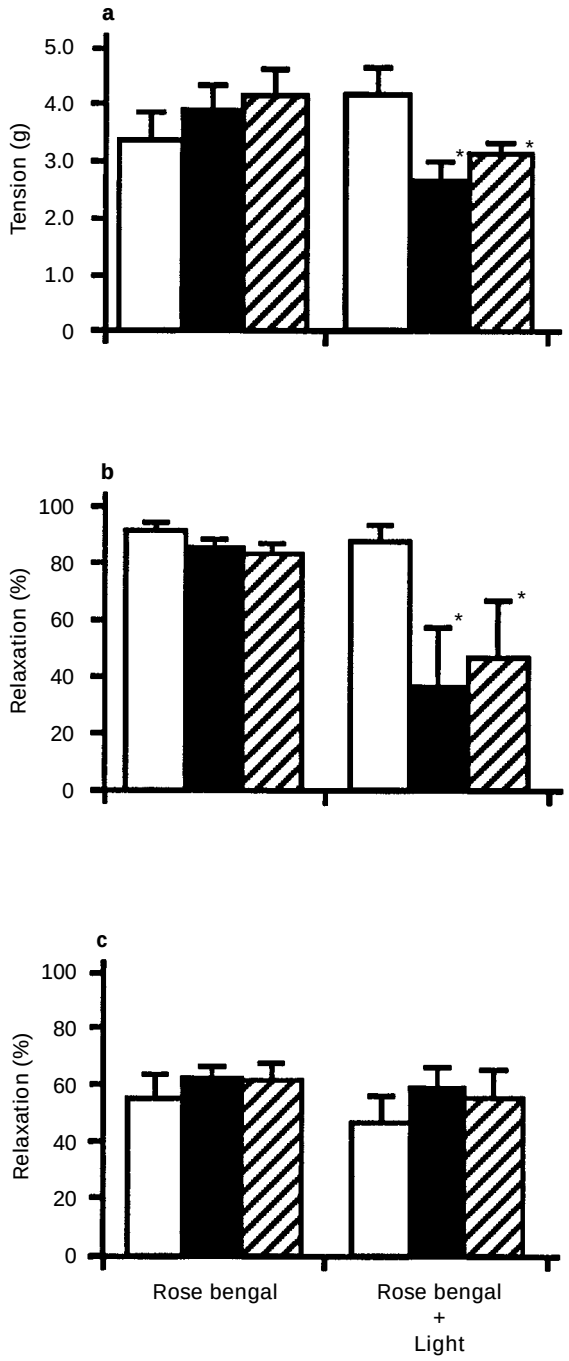

Figure 4 The effect of rose bengal with illumination for 30 min on the NA (a)-, ACh (b)- and nitroglycerin (c)-induced responses in rabbit mesenteric artery ring preparations. The experimental conditions were similar to those described in Figure 3 except that 10^{-6} M nitroglycerin was used and the relaxation response to nitroglycerin was determined in a manner similar to that of ACh. The NA-, ACh- and nitroglycerin-induced responses in time-matched and illumination control rings were stable during the experimental period; therefore, the data of time-matched control and illumination control values are not presented. First test values (open columns) were used as controls, and compared with the values of 2nd test (solid columns) and recovery responses (hatched columns). Relaxation responses to ACh (b) and nitroglycerin (c) are expressed as percentage of stable plateau tension induced by the addition of NA. Each column represents the mean, and vertical lines show s.e.mean ($n = 5$); n refers to the number of rabbits from which the mesenteric artery was taken. $^*P < 0.05$: significantly different from corresponding control values.

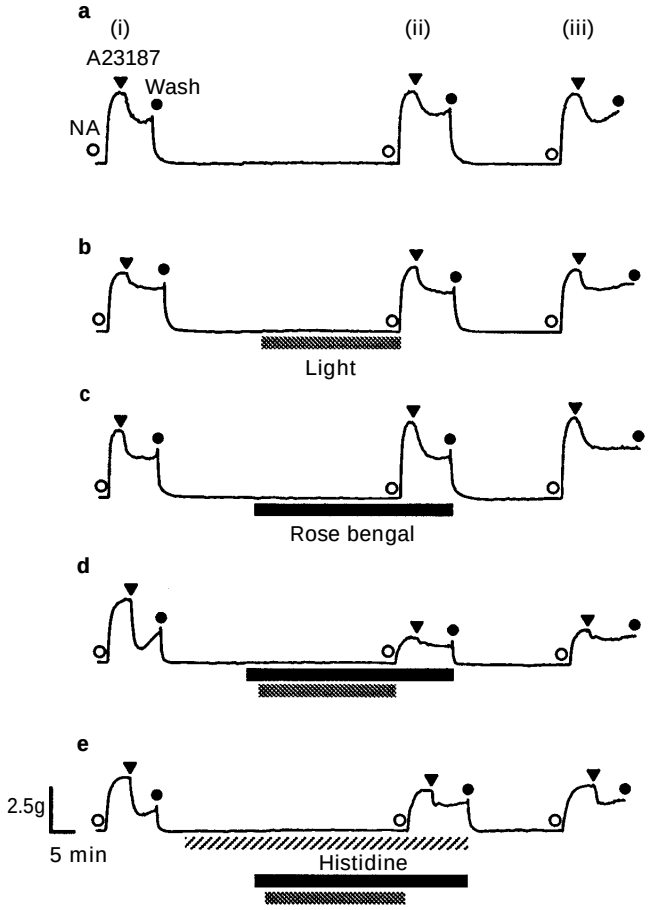

Figure 5 The effect of rose bengal and/or illumination for 30 min on the calcium ionophore A23187-induced relaxation responses of rabbit mesenteric artery preparations, and effect of histidine. The experimental conditions were essentially similar to those described in Figure 3 except that histidine (25 mM) was used and relaxation responses to 10^{-7} M A23187 were determined in the absence (i) and presence (ii) of rose bengal; recovery responses to A23187 (iii) were also determined. Histidine was added 15 min before the addition of rose bengal. Five ring preparations (for time-matched control (a); illumination control (b); rose bengal alone (c); rose bengal plus illumination (d) and rose bengal plus illumination in the presence of histidine (e)) obtained from the same vessel were studied in parallel. Tracings are representative of three experiments.

Table 1 Effects of various reactive oxygen species scavengers on NA- and ACh-induced responses with rose bengal (50 nM) and illumination (6,000 lux, for 30 min)

Experimental addition	*NA contraction (g)*			*ACh relaxation (%)*		
	T1	*T2*	*T3*	*T1*	*T2*	*T3*
Histidine	3.30±0.23	4.25±0.21*	3.73±0.31	87.43±10.65	76.44±13.30	86.60±4.91
Rose bengal+light	3.42±0.32	1.28±0.13**	1.85±0.35**	93.31±3.65	16.82±10.05**	21.01±9.91**
Histidine+rose bengal+light	3.13±0.64	4.03±0.47¶¶	3.13±0.75¶	92.61±2.81	65.79±11.47¶¶	80.11±13.21¶¶
SOD	3.60±0.70	3.55±0.53	3.85±0.56	91.10±4.37	89.29±4.29	88.34±5.06
Rose bengal+light	3.62±0.87	1.77±0.63**	2.33±0.74*	91.47±2.93	11.11±11.11**	18.52±18.50**
SOD+rose bengal+light	3.70±0.12	1.67±0.17**	2.40±0.40*	88.56±4.59	0**	5.42±2.92**
Catalase	2.90±0.27	3.43±0.31	3.05±0.33	94.12±1.34	97.32±1.92	96.33±1.68
Rose bengal+light	2.25±0.34	1.00±0.35**	1.73±0.43*	99.19±0.81	51.04±17.78**	64.68±11.44**
Catalase+rose bengal+light	3.03±0.56	2.10±0.66**	2.30±0.56*	94.58±2.81	55.01±13.11**	56.20±14.74**
DMTU	4.48±0.38	4.46±0.46	4.88±0.46	73.63±8.03	69.55±9.39	68.84±8.51
Rose bengal+light	4.19±0.48	2.71±0.60**	3.51±0.58*	67.96±12.52	16.67±9.62**	20.67±10.59**
DMTU+rose bengal+light	4.56±0.92	3.15±1.01*	3.84±0.90*	67.86±16.43	26.79±14.14**	32.61±17.62**

The experimental conditions were essentially similar to those described in Figure 3 except that reactive oxygen species scavengers were used; histidine (25 mM), sod (33 units ml^{-1}), catalase (32 units ml^{-1}) or DMTU (10 mM) was added 15 min before the addition of rose bengal or added in a time-matched manner (in rings treated with scavenger alone). Four ring preparations (for scavenger alone, rose bengal plus illumination in the presence or absence scavenger, and time-matched control) obtained from the same vessel were studied in parallel; the ability of the ring preparations to respond to photolysed rose bengal was confirmed in each experimental protocol. The NA- and ACh-induced responses in time-matched control rings were stable during experimental period; therefore, the data of time-matched control values are not presented. First test values (T1) were used as controls, and compared with the values of 2nd test (T2) and recovery responses (T3). Relaxation responses to ACh (10^{-6} M) are expressed as percentage of stable plateau tension induced by the addition of NA (2.58×10^{-6}). Data represent the mean±s.e.mean ($n=3$ to 4); n refers to the number of rabbits from which the mesenteric artery was taken. *$P<0.05$; **$P<0.01$: significantly different from corresponding control values; ¶$P<0.05$; ¶¶$P<0.01$: significantly different from corresponding value for rose bengal plus light.

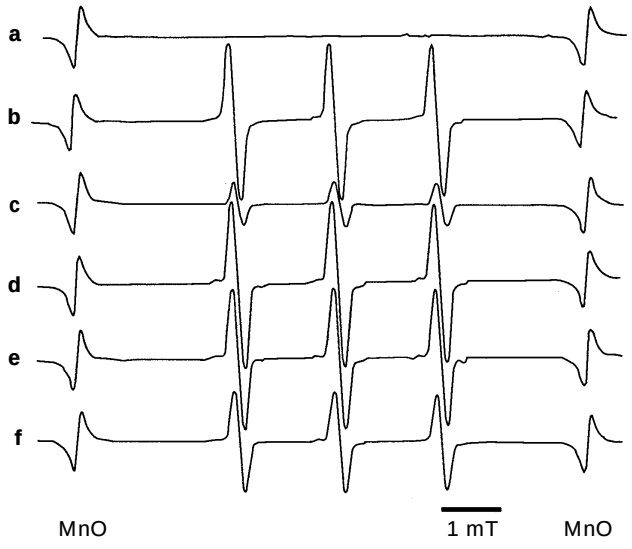

Figure 6 Effects of various reactive oxygen species scavengers on the e.s.r. spectrum of photolysed rose bengal in the presence of TEMP. Rose bengal (50 nM) was illuminated (6,000 lux) for 30 min in the presence of TEMP (40 mM). At the end of illumination, e.s.r. spectrum was recorded as described in Methods. Reaction mixture was the same as that of the vessel reactivity experiment except that the ring preparation was omitted. (a) Shows incubation of TEMP with rose bengal without illumination, (b) photolysed rose bengal with TEMP and (c) photolysed rose bengal with TEMP plus histidine (25 mM), (d) SOD (33 units ml^{-1}), (e) catalase (32 units ml^{-1}) or plus (f) DMTU (10 mM). The scavengers used were added 15 min before the addition of rose bengal; rose bengal was added 2 min before the illumination. Signals appearing at both sides of the e.s.r. spectra correspond to Mn^{2+} (MnO) installed in the e.s.r. cavity as a reference.

Figure 7b shows that photolysis of rose bengal formed a 1:2:2:1 quartet (the hyperfine splittings were $A_N=A_H^{\beta}=1.49$ mT), characteristic of the HO·-DMPO spin adduct (Zweier, 1988). This signal was effectively blunted by the HO· scavenger DMTU (Figure 7c), $O_2\cdot^-$ scavenger SOD (Figure 7d) and H_2O_2 scavenger catalase (Figure 7e), suggesting that photolysed rose bengal can produce HO· radical via generation of other reactive oxygen species such as $O_2\cdot^-$ and H_2O_2 in our system. Histidine had no effect on this system (Figure 7f).

Discussion

Reactive oxygen intermediates can evoke both vasodilatation and vasoconstriction either by a direct or indirect action on vascular smooth muscle (see Rubanyi, 1988; Katusic & Vanhoutte, 1989). These varied responses may be dependent on the type and amount of radical species present. Singlet oxygen is one of the oxygen species produced during ischaemia and reperfusion (Kukreja *et al*., 1993). A series of studies from Hearse's laboratory has demonstrated that maximal damage is induced by $^1O_2$ to the blood vessels, which may lead to the progressive impairment of blood flow (Kusama *et al*., 1989; Vandeplassche *et al*., 1990). It is likely that $^1O_2$ directly attacks endothelial cells or smooth muscles, thus causing vascular incompetence. Hence, the present study was aimed to determine whether $^1O_2$ was capable of attenuating vascular reactivity *in vitro* and consequently to establish the possible mechanism by which $^1O_2$ damages endothelium-dependent relaxation and smooth muscle contraction produced by ACh and NA, respectively, in rabbit mesenteric artery. On the basis of the present findings, we conclude that exogenous $^1O_2$ generated from photolysed rose bengal selectively damages endothelium-dependent relaxation as opposed to endothelium-independent relaxation, and that $^1O_2$ depresses NA-induced contraction in part via oxidant-induced receptor dysfunction. This, in turn, leads to vascular incompetence. Histidine exerted a powerful protection against $^1O_2$-mediated damage to the responses to ACh and NA.

It has been shown that the quenching of rose bengal by oxygen leads to the production of approximately ~75% $^1O_2$ and ~20% $O_2\cdot^-$ (Lee & Rodgers, 1987). In our experimental system, it was confirmed that photoactivation of rose bengal leads not only to the generation of $^1O_2$ and $O_2\cdot^-$ but to that of H_2O_2 and HO·, which might impose an oxidant stress on the vascular preparations; thus account for the vascular dysfunction. Our studies have shown that a variety of scavengers (SOD, catalase and DMTU), which should eliminate

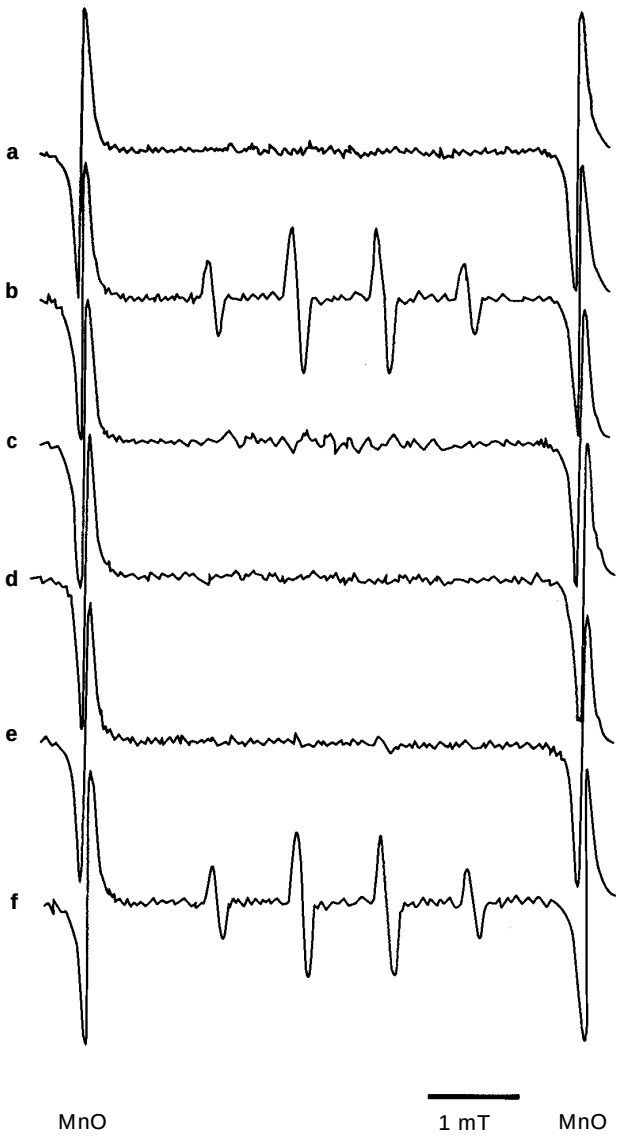

Figure 7 The e.s.r. spectra of photolysed rose bengal in the presence of DMPO. Reaction conditions were identical to those of Figure 6 except that DMPO (100 mM) was used as the spin trap. (a) Shows incubation of DMPO with rose bengal illumination, (b) photolysed rose bengal with DMPO, and (c) photolysed rose bengal with DMPO plus DMTU, (d) SOD, (e) catalase or plus (f) histidine. Signals appearing at both sides of the e.s.r. spectra correspond to Mn^{2+} (MnO) installed in the e.s.r. cavity as a reference.

$O_2\cdot^-$, H_2O_2 and HO·, had no effect on the attenuated responses to NA and ACh induced by photolysed rose bengal (Table 1). In contrast, the inhibition of the effect of photolysed rose bengal (2nd test) and recovery responses to NA and ACh (3rd test) were obtained with addition of histidine, a $^1O_2$ quencher (Table 1). Although the evidence for $^1O_2$ production is conclusive and the protective effect of histidine is specifically attributed to $^1O_2$ scavenging (Figure 6), the formation of $O_2\cdot^-$, H_2O_2 and HO· by photolysis of rose bengal is still in question. Spin trapping of photolysed rose bengal showed clearly the formation of HO·-DMPO adduct (Figure 7), which was inhibited by SOD, catalase and DMTU. The $O_2\cdot^-$ generated from photolysed rose bengal then forms additional reactive oxygen intermediates such as H_2O_2 and HO· by the Haber-Weiss or Fenton reaction. Thus SOD appeared to eliminate the HO·-DMPO signal due to scavenging of $O_2\cdot^-$ and catalase reduced the signal via scavenging H_2O_2, the precursor of HO· in this system. These results suggest that there was formation of $O_2\cdot^-$, H_2O_2 and HO· in addition to $^1O_2$ by photolysis of rose bengal. However, the observed vascular dysfunction is not caused by $O_2\cdot^-$, H_2O_2 and HO·; this is shown by the inability of SOD, catalase and DMTU to protect the effect of illuminated rose bengal (Table 1). Taken together, it is highly likely that it may be $^1O_2$ rather than $O_2\cdot^-$, H_2O_2 or HO· that is responsible for the vascular dysfunction induced by photolysed rose bengal.

The present study has shown that $^1O_2$ generated from photolysed rose bengal produced marked attenuation of endothelium-dependent relaxation caused by ACh, but not endothelium-independent relaxation caused by nitroglycerin. When the vessel rings were thoroughly rinsed after treatment with photolysed rose bengal, the response to ACh was not restored. The lack of endothelial response after exposure to $^1O_2$ may be due to damaged endothelium or inhibition of endothelium-derived relaxing factor (EDRF) synthesis, but not due to the direct inactivation of EDRF(s). Both EDRF(s) and nitroglycerin have been shown to produce vascular relaxation by increasing the production of guanosine 3′:5′-cyclic monophosphate (cyclic GMP), which inhibits the smooth muscle contractile response (Rapoport & Murad, 1983). Because previous exposure to $^1O_2$ generated from photolysed rose bengal only inhibits the action of endothelium-dependent vasodilators, such as the muscarinic receptor agonist ACh (Figure 4b), and the calcium ionophore A23187 (Figure 5), which causes relaxation without acting via receptors (Singer & Peach, 1982), and not that of nitroglycerin (Figure 4c), its action may be at some site(s) between the endothelial site of interaction and the smooth muscle production of cyclic GMP, but not at the receptor site; the susceptibility of the processes of production of cyclic GMP mediated by nitroglycerin in smooth muscle cells to $^1O_2$ may differ greatly from that mediated by EDRF(s). Thus it is likely that $^1O_2$ may selectively damage endothelial function.

Why $^1O_2$ attacked endothelium and inhibited EDRF(s) synthesis processes rather than the process of cyclic GMP production mediated by nitroglycerin is not clear. This may be due to the fact that there is an active site in EDRF(s) synthesis processes, possibly, Ca^{2+}-calmodulin dependent process (Okabe *et al.*, 1987; 1989), which may be specifically vulnerable to $^1O_2$. Because of the good permeability of rose bengal across cell membranes and formation of $^1O_2$ in cells upon illumination, it is reasonable to assume that the site of action exerted by $^1O_2$ is intracellular. Although HO· radicals may selectively damage endothelium-dependent relaxation of canine coronary artery (Todoki *et al.*, 1992), our data suggest yet another species of reactive oxygen such as $^1O_2$ may be the primary mediator of endothelial dysfunction because DMTU, a compound that is more effective than traditional HO· scavengers and is highly cell permeable (Fox *et al.*, 1983; Fox, 1984), has no effect on the observed attenuation of the relaxation caused by ACh (Table 1). Therefore, HO· generated from photolysed rose bengal in cells is not an active agent on the endothelium-dependent relaxation.

Although our data clearly establish a role of $^1O_2$ in the photolysed rose bengal-induced loss of vasoconstrictor effectiveness of NA, the cellular mechanism involved in the $^1O_2$-mediated vascular smooth muscle damage is unclear from the present data. Potential sites of damage include α-adrenoceptors, alterations in second messenger function, defects in Ca^{2+} mobilization, and disruption of contractile protein function. However, our observation that the pD_2 decreased without a significant reduction in maximum tension generation (Figure 2b) is consistent with the notion that $^1O_2$-induced receptor dysfunction may play a more important role. The contractile state of vascular smooth muscle is dependent on cytoplasmic Ca^{2+} concentration that in turn is dependent on release and uptake of intracellular Ca^{2+} stores and the flux of extracellular Ca^{2+} across the cell membrane. Vascular incompetence may be reflected by disturbances of Ca^{2+} homoestasis. This is confirmed by the cytochemical studies in which $^1O_2$ caused con-

spicuous redistribution of deposition of Ca^{2+} in heart (Vandeplassche *et al*., 1990). Furthermore, Freas *et al*. (1991) have been postulated that reactive oxygen species act indirectly on tension development by changing the permeability of the vascular smooth muscle membrane to Ca^{2+} and thus contracting vascular smooth muscle. This is not suggested by our observations, as exposure of the vascular preparations to $^1O_2$ did not produce contractions. Another possibility is that the observed attenuation of NA-induced contraction of the vascular preparations caused by $^1O_2$ might be produced by a reduced influx of Ca^{2+}, possibly through the voltage-dependent Ca^{2+} channels, or by reduced release of Ca^{2+} from intracellular Ca^{2+} stores. Since we did not determine the effect of $^1O_2$ on this system, we cannot rule out such a possibility. Apparently, further investigation is necessary to examine the role of disturbances of Ca^{2+} homeostasis in attenuating NA-induced contraction by $^1O_2$ and to discriminate its role from that played by α-adrenoceptor dysfunction.

It remains unclear whether upon illumination, rose bengal also generates $O_2\cdot^-$ and H_2O_2 within cells. If this occurs and if these reactive oxygen species modify endothelial and vascular functions by intracellular mechanisms, SOD and catalase which do not cross cell membranes, should not exert a protective effect. Furthermore, it is difficult to prove that the effect exerted by histidine described here is intracellular, although, in photodynamic terms, it is inevitable that $^1O_2$ is formed under these conditions. In the present studies, we have attributed the vascular injury to $^1O_2$ rather than $O_2\cdot^-$ and H_2O_2. This decision was based on a previous study (Todoki *et al*., 1992) in which we demonstrated that dihydroxy fumarate-derived $O_2\cdot^-$ and H_2O_2 (300 μM), added exogenously, were without effect on endothelium-dependent relaxation. Finally, our observation (Ishibashi *et al*., 1996) that the amount of H_2O_2 generated from photolysed rose bengal was ~90 μM led us to conclude that $^1O_2$ is the most likely culprit. However, whether the site at which rose bengal accumulates is the endothelium or the smooth muscle or whether histidine binds to cell membranes or is taken up into the intracellular compartment remains to be resolved.

Our studies did not test the direct inactivation of EDRF(s) and NA by $^1O_2$, since the vasodilators that mediate release of EDRF(s) and NA were given after photolysis of rose bengal to the tissue bath.

In summary, the data presented here convincingly show that $^1O_2$ is one of the most destructive oxygen species. Endothelium-dependent relaxation was extremely vulnerable to $^1O_2$. Singlet oxygen also depresses NA-induced contractions possibly via α-adrenoceptor dysfunction. Histidine was shown to be one of the most powerful agents available to preserve the vascular reactivity from $^1O_2$-mediated damage.

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